

Potential limitations of the *Sleeping Beauty* transposon use in gene expression studies*

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MCPIP2 is the least known member of the MCPIP family of proteins. Recently we have found that it is a new RNase involved in transcript turnover. However, the full spectrum of its cellular targets is still unidentified. To discover transcripts which are regulated by this protein we have employed *Sleeping Beauty* transposons. This tool allows for rapid generation of a stable transgenic cell line with inducible expression of the desired gene. In this study, we analysed how the *Sleeping Beauty* system itself influences expression of chosen genes, namely IL-6, Regnase-1 and VEGF. We found that the system alone may influence expression of IL-6. Our results indicate that *Sleeping Beauty* transposons should be used with caution in studies that are focused on changes in the transcript level.

Key words: transcriptome analysis, *Sleeping Beauty* transposons, inducible gene expression

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Abbreviations: AAV, adeno-associated virus; cGAS, cyclic GMP-AMP synthase; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DAI, DNA-dependent activator of interferon-regulatory factors; Dox, doxycycline; DMEM, Dulbecco's Modified Eagle Medium; dsRNA, double stranded RNA; GOI, gene of interest; GFP, green fluorescent protein; gRNA, guide RNA; HDR, homologous recombination; IL-6, Interleukin 6; ITR, inverted terminal repeats; Luc, luciferase; MCPIP, monocyte chemoattractant protein-induced protein; miRNA, micro RNA; mRNA, messenger RNA; NHEJ, non-homologous end joining; PCR, polymerase chain reaction; Reg-1, Regnase 1; RNAseq, RNA sequencing; SB system, *Sleeping Beauty* system; sgRNA, single guide RNA; siRNA, silencing RNA; TALEN, Transcription Activator-Like Effector Nuclease; TRE, tetracycline response element; VEGF, vascular endothelial growth factor

INTRODUCTION

Capability to modulate expression of a specific gene has been beneficial to understand its function. Since discovery of the RNA interference phenomenon in 1998, double-stranded RNAs (dsRNAs), such as long dsRNAs, siRNAs, and microRNAs, have been used as research tools to down-regulate an mRNA level by its cleavage, degradation or translational repression (the last one mainly through miRNA) (Lam *et al.*, 2015). However, delivery of exogenous dsRNAs in mammalian cells

can be associated with unintentional activation of pattern recognition receptors of the innate immune system and induction of the interferon pathway (Gantier & Williams, 2007). As an effect, unwanted changes in the level of different genes' induction are observed and can be the source of false conclusions. Recently, new tools for genome editing, such as Zinc Finger Nucleases, TALENs (Transcription Activator-Like Effector Nuclease) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas have emerged (Gaj *et al.*, 2013). The first two employed complicated protein-DNA interactions for targeting the gene of interest (GOI), while the CRISPR-Cas system uses small RNAs with an on-target specificity. All three systems generate double-stranded DNA breaks which can be repaired through non-homologous end joining (NHEJ) or homologous recombination (HDR). In the course of NHEJ, insertions or deletions can be created which in turn may result in frame-shift mutations and generation of premature STOP codons. HDR-mediated repair is more predictable than NHEJ, however, it occurs with lower frequency and additionally a template for recombination has to be delivered into the cell (Liu *et al.*, 2019). The biggest threat concerning the use of the known tools for genome editing is their potential to induce unwanted, off-target effects. This disadvantage also applies to RNAi technology.

An opposite approach for studying a gene's function is its overexpression. Two types of vectors are the most frequently used for efficient delivery of transgenes, non-viral (plasmids) and viral vectors. When plasmids are employed as a delivery vehicles, transcription of the transgene proceeds with an efficiency depending on the type of the promoter present in the plasmid. Viral promoters orchestrate expression of a transgene at a very high level which quickly diminishes over several days. Use of eukaryotic promoters results in longer and moderate expression of a given transgene. Maintaining prolonged selection pressure can lead to integration of the expression vector into the genome. However, this is a rare event and prone to artifacts (Zucca *et al.*, 2013). DNA delivery to mammalian cells can be also propagated by viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses (AAVs), herpesvirus and poxvirus. Retroviral vectors can stably integrate into the genome of the infected cell but require cell division for transduction. Cell division is not needed during infection with adenoviruses and herpes virus but there is no integration into the host genome and the expression of the transgene is transient. AAVs also infect many non-dividing and dividing cell types but have a limited DNA capacity (4 kb). Poxvirus vectors have high capacity but exert potential cytopathic effects (Kotterman *et al.*, 2015; Walther & Stein, 2000).

Most of the known plasmid vectors suffer from the limited duration of transgene expression. Lack of genomic insertion causes cellular degradation and/or dilution of the vector. An additional problem concerns the cellular toxicity of the transfection process. This is mainly related to the high concentration of recombinant proteins which results in activation or overloading of specific biological pathways or in aggregate creation of recombinant proteins (Vavouri *et al.*, 2009). Therefore, despite the fact that cell transfection had been carried out since the 1990s, the need for better tools for transgene overexpression is still persisting.

The potential of transposable elements for genome modification has been analyzed since the nineties. These natural elements have the ability to move within the genome through a cut-and-paste mechanism. The DNA transposons contain a transposase gene flanked by inverted terminal repeats (ITRs) which carry the transposase binding sites. Transposase catalyzes excision of the transposon from its original location and promotes its translocation. Importantly, it is possible to separate both functional components of transposons. Any GOI flanked by ITRs can undergo transposition in the presence of a transposase supplied *in trans*. The synthetic *Sleeping Beauty* transposon is an equivalent to an ancient Tc1/mariner-like element present in the salmonid fish genome. Given that this element was inactive for 10 million years and then reactivated in 1997, it was named *Sleeping Beauty* (SB), after the famous fairy tale. The SB has been generated by “reverse engineering” from defective copies of an ancestral transposon in fish. Its reactivation included homology comparison in combination with site-directed mutagenesis. Additionally, its transpositional activity was enhanced by obtaining a hyperactive mutant of SB transposase, named SB100x (Kowarz *et al.*, 2015; Kebriaei *et al.*, 2017; Hudecek *et al.*, 2017). Advantages of the SB system include permanent genomic insertion and the ability to maintain and propagate transposon vectors as plasmid DNA, meaning simple and inexpensive manufacture. Another advantage of the SB system is the target site specificity. Integration of SB transposons occurs at sequences containing TA-dinucleotides. There are about 30 000 such sequences in the human genome. This is an important improvement of SB system over viral vectors which integrate into the genome at promoters and first introns of actively transcribed genes. The SB system is also better for gene delivery than viral vectors in terms of its lower immunity which is a significant factor for *in vivo* studies (Kowarz *et al.*, 2015).

Previously, we have found that MCP2 is involved in regulation of inflammation through destabilization of transcripts of proinflammatory cytokines (Wawro *et al.*, 2019). To identify new RNA molecules which are regulated by MCP2, we employed the SB system for overexpression of MCP2 in a human astrocytoma cell line, U251-MG. Surprisingly, we found that the SB system alone influences expression of some genes. The aim of this work was to analyse the use of the *Sleeping Beauty* tool for gene expression studies.

MATERIALS AND METHODS

Cell culture. Human astrocytoma U251-MG (ECACC 09063001), formerly known as U373-MG cell line, was used in experiments. It was derived from a 75-year old male patient with malignant glioblastoma multiforme tumour by an explant technique, and established at the Wallenberg laboratory, Uppsala, Sweden (Westermarck *et*

al., 1973). It was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose (BioWest, Nuaille) supplemented with 10% (v/v) Fetal Bovine Serum Tetracycline free (BioWest, Nuaille), at 37°C in a humidified atmosphere with 5% CO₂.

Construct preparation. The pSBtet-GP-MCPIP2 construct was prepared by cloning ZC3H12B coding region (gene encoding MCP2, NM_001010888.3) into the Sleeping Beauty vector pSBtet-GP (Addgene, #60495). PCR products were amplified from a previously prepared vector pcDNA5/FRT/TO-SF-ZC3H12B, containing ZC3H12B coding region fused with Strep-TagII and FLAG tags using Q5 High Fidelity DNA Polymerase (New England Biolabs, Ipswich) (Wawro *et al.*, 2019). The following primers were used:

forward: AGGCCTCTGAGGCCACCCACCATGGCAAGCTGGAGC,
reverse: AGGCCTGACAGGCCTCAACGTGCAGCCCTAAGC

After gel electrophoresis, PCR products were excised, purified (Gel/PCR ME Mini Kit, Syngen, Wrocław), cleaved with the SfiI restriction enzyme (New England Biolabs, Ipswich), and purified after enzymatic reaction. Then, products were ligated into gel-purified pSBtet-GP vector linearized with the SfiI enzyme by using a T4 DNA ligase (Thermo Fisher Scientific, Waltham). Obtained constructs were verified by restriction analysis and sequencing.

Transfection and cell selection. U251-MG cells were seeded in 12-well plates at a density of 1×10⁵ cells/well. 24 hours later, the cells were transfected with the pSBtetGP-MCPIP2 vector and the pCMV(CAT)T7-SB100 vector (Addgene, #34879), using the TransIT-LT1 Transfection Reagent (Mirus, Madison) according to the manufacturers' instructions. The total amount of 1 µg of DNA per well was used in a ratio of 9:1 (pSBtet-GP: pCMV(CAT)T7-SB100). The next day, the cells were transferred to a 6-well plate and puromycin (Invivogen, San Diego) was added to a final concentration of 1 µg/ml. After a week of selection, stable cell line was generated. Transfection efficiency was assessed by observation of GFP fluorescence under a microscope.

RNA isolation and cDNA preparation. Total RNA was isolated from U251-MG cells modified with the *Sleeping Beauty* system using the Chomczynski method (Chomczynski & Sacchi, 2006). The RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham). 1 µg of RNA was treated with RQ1 RNase-free DNase (Promega, Madison) according to the manufacturer's instructions, and then reverse-transcribed to cDNA with M-MLV-Reverse transcriptase (Promega, Madison) and 500 ng of oligo(dT)₁₅ primers (Genomed, Warsaw) according to the manufacturer's instructions.

Real-time PCR. Real-time PCR was performed using SYBR-A RT HS-PCR Mix (A&A Biotechnology, Gdynia) and primers specific to analyzed transcripts (Genomed, Warsaw):

IL-6: forward: GACAGCCACTCACCTCTTCA
reverse: AGTGCCCTCTTTGCTGCTTTC
Regnase-1: forward: GGAAGCAGCCGTGTCCCTATG,
reverse: TCCAGGCTGCACTGCTCACTC
VEGF: forward: ATGCGGATCAAACCTACCAAGGC
reverse: TTAAGCTCAAGCTGCCTCGCCTTGC
EF-2: forward: GGTGCAGTGCATCATCGAGGAGTC,
reverse: TCGCGGTACGAGACGACCGG

mRNA level in each sample was analyzed in duplicate, and the results were normalized to the reference gene (EF-2). The relative level of transcripts was calculated by using the $\Delta\Delta C_T$ method.

Western Blot analysis. U251-MG cells with inducible overexpression of MCPIP2 were plated in a 12-well plate. The following day, protein expression was induced by adding doxycycline to a final concentration of 1 $\mu\text{g}/\text{ml}$. 24-h after induction, induced cells as well as non-induced control cells were lysed in 100 μl /well of Laemmli lysis buffer (0.35 M Tris-HCl, 35% (v/v) glycerol, 10% (w/v) SDS, 3.6 M β -mercaptoethanol, 0.12 g/ml bromophenol blue) and denatured at 95°C for 7 minutes. 20 μl of samples were separated by SDS-PAGE and wet transfer was performed onto PVDF membrane (Merc Millipore, Burlington). After transfer, the membranes were blocked in 5% (w/v) non-fat dry milk in TTBS (20 mM Tris, 150 mM NaCl, 0.1% Tween-20), transferred to an antibody solution, and incubated overnight at 4°C with gentle agitation. The following primary antibodies were used: anti-FLAG (D6W5B, 1:2000 Cell Signaling, Danvers) or anti- β -actin (13E5, 1:2000 Cell Signaling, Danvers). After primary antibody incubation, the membranes were washed three times for 5 minutes at room temperature with TTBS, and then incubated with HRP-conjugated secondary antibodies (anti-rabbit, 1:10000, Cell Signaling, Danvers) for 1 hour at room temperature with gentle agitation. Then, the membranes were washed three times with TTBS (as previously), incubated with Clarity Western ECL Blotting Substrate (BioRad, Hercules) for 5 minutes at room temperature, and chemiluminescence was detected using Fusion-Fx system (VilberLourmat, Marne-la-Vallée).

Generation of a knockout cell line and analysis of mutations. U251-MG cells were modified using CRISPR/Cas9 method targeting the *ZC3H12B* gene to generate a cell line without MCPIP2 expression (U251-MG-M2-KO). Cas9 mRNA was transcribed in vitro using the px330 plasmid (Addgene #42230) linearized with XbaI (New England Biolabs, Ipswich) as a template, according to standard mRNA synthesis protocol (New England Biolabs, Ipswich, E2060). Next, cells were transfected with synthesized Cas9 mRNA together with sgRNA targeting *ZC3H12B* (CRISPR961504_SGM, Thermo Fisher Scientific, Waltham) in 4 hour interval using Messenger Max and RNAiMAX (Thermo Fisher Scientific, Waltham), respectively, according to the manufacturer's instructions. After transfections, the cells were seeded in 96-well plates at a 0.5 cell/well density to obtain single cell clones. Presence of a mutation in the desired gene was analyzed using the mismatch sensitive nuclease Cell (Qiu *et al.*, 2004). Genomic DNA was isolated from the obtained cell clones and wild type cells by lysis in Proteinase K lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton-X supplemented with 1 $\mu\text{g}/\mu\text{l}$ of Proteinase K) and PCR was performed using primers flanking the targeted DNA region. The following primers were used: forward: CCG-CATGTGCTTTTCAGGAG, reverse: ACCAGGCT-TACCTCATTGCC. PCR products were reannealed by heating to 95°C and gradual cooling to 4°C. In addition, reannealing of each clone with products obtained with gDNA from wild type cells was performed. In the next step, two reactions were prepared for each tested clone: by mixing PCR product itself (4 μl), or by mixing PCR product (2 μl) with the product obtained with wild type gDNA (2 μl). To each reaction, 6 μl of 1×Cell buffer was added. Heteroduplexes were cleaved by adding 0.5 μl /reaction of the mismatch sensitive nuclease Cell

that recognizes single, unpaired nucleotides and performs cleavage. Cell and Cell buffer were prepared as described by Till and others (Till *et al.*, 2006). Products of cleavage were separated in 1% agarose gel (w/v) in 1×TAE buffer supplemented with 5 $\mu\text{g}/\text{ml}$ of ethidium bromide and presence of the mutation was analyzed. Presence of mutation in selected clones was also confirmed by sequencing (Genomed, Warsaw).

Statistical analysis and graphs. Statistical analysis and graphs were performed using GraphPad Prism (v. 5.0, GraphPad Software Inc.). Scheme of expression cassettes was obtained by using Adobe Illustrator CC (2019). Detailed information about statistical tests is indicated in figure descriptions.

RESULTS AND DISCUSSION

Generation of a U251-MG cell line with MCPIP2 knockout

To maximize the difference between transcriptomes from cells with overexpression of MCPIP2 and control (reference) cells, we have generated a U251-MG cell line with MCPIP2 gene knockout (U251-MG-M2-KO cells) by the CRISPR-Cas9 system. We decided to use the all-RNA approach in which Cas9 and chemically modified gRNA are delivered into cells in an RNA form. In comparison with unmodified gRNA, synthetic gRNA shows improvement in stability and increased efficiency of genome editing when Cas9 mRNA is co-delivered into the cells (Hendel *et al.*, 2015). This approach is also less toxic than the classic approach. In cells transfected with plasmid DNA the Cas9 protein accumulates over time, whereas in Cas9 mRNA transfected cells the protein level is relatively low with the greatest peak four hours post-transfection. Because of this features, off-target activity for the all-RNA approach is lower than that of plasmid DNA transfection (Ranganathan *et al.*, 2015). Using the all-RNA approach we were able to obtain a stable cell line with *ZC3H12B* gene editing, confirmed by the Cell nuclease assay and sequencing (Fig. 1). After transfection with Cas9 mRNA and sgRNA, the cells were seeded into a 96-well plate to obtain single cell clones. The clones were tested using the Cell mismatch sensitive nuclease to discriminate whether a mutation aroused, as well as to determine if one, or both gene copies were altered (Fig. 1A). To do that, PCR was performed with DNA obtained from mutants as well as with wild type DNA. Then, mutant PCR products were reannealed, and additional reaction was performed at the same time for each clone by annealing equal amounts of mutant and wild type PCR products. After reannealing, heteroduplexes were subjected to Cell cleavage, and products were visualized by gel electrophoresis. In case of heteroduplexes from mutant PCR, if cleavage products are present, a mutation occurred only in one allele (heterozygote, +/-). However, absence of cleaved products can represent two situations: no mutation (wild-type, +/+) or mutation present in both alleles (homozygote, -/-). To discriminate between these two options, additional annealing reaction with wild-type PCR is necessary. In this situation, if cleavage products are present we can assume that the mutation occurred in both gene copies (Fig. 1A). In our case, we obtained three clones that harbor mutation in both alleles (K5, K7, K12, Fig. 1B). Sequencing revealed that only in the genome of clone K5 the CRISPR/Cas9 cleavage caused a deletion of a single nucleotide that leads to a frame-shift mutation and generation of a pre-

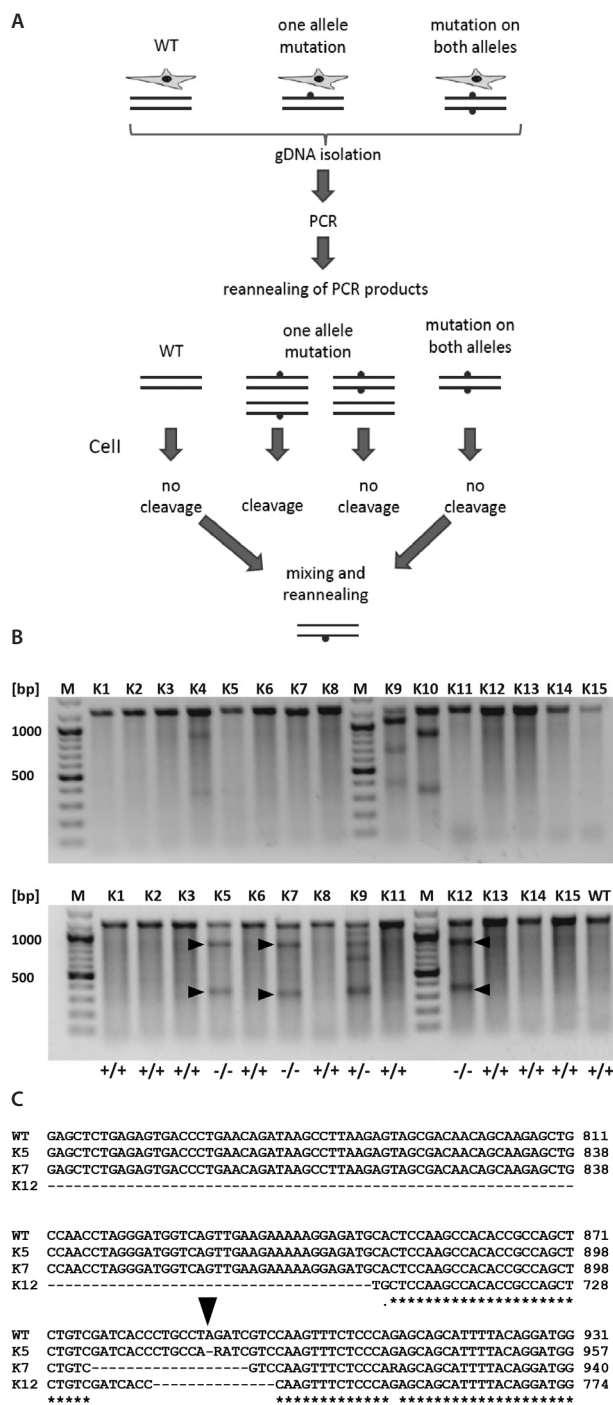


Figure 1. Analysis of mutation in the *ZC3H12B* gene with Cell mismatch sensitive nuclease.

(A) Schematic representation of mutation analysis using the Cell mismatch sensitive nuclease. (B) Analysis of mutants with the use of Cell nuclease. PCR was performed with gDNA isolated from mutants (K1-K15), as well as wild type U251-MG cells (WT). Mutant PCR products and mixtures of mutant and wild type PCR products were reannealed, and cleavage with the Cell nuclease was performed. Upper panel: cleavage of mutant PCR products after reannealing. Lower panel: cleavage of heteroduplexes of WT PCR product and PCR products from the upper panel. K1-K15, analysed clones. Arrows on the lower panel indicate products of Cell cleavage. “-/-”, “+/-”, “+/+” indicate homozygote, heterozygote or wild type genotype, respectively. (C) Alignment of wild type U251-MG (WT) and K5, K7, K12 mutant DNA fragments after CRISPR/Cas9 genome editing targeting *ZC3H12B*. Image presents fragment of the *ZC3H12B* sequence where mutation occurs. “-” indicates deleted nucleotides. Black arrow indicates the position of nucleotide deleted in K5, leading to a frame-shift.

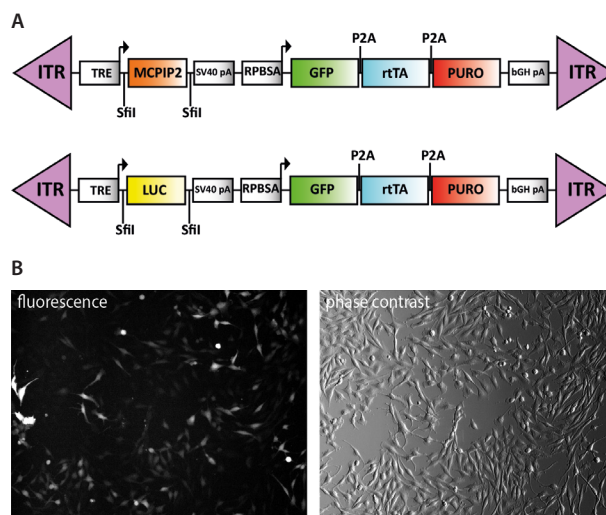


Figure 2. Generation of U251-MG cell line with the *Sleeping Beauty* transposon-based inducible overexpression of MCPIP2 protein.

(A) Backbone of pSBtet-GP and pSBtet-GP-MCPIP2 vectors used for generation of the U251-MG-LUC and U251-MG-M2-SB cell lines, respectively. ITR, inverted terminal repeats; TRE, inducible promoter with tetracycline response element; MCPIP2, coding sequence for MCPIP2 with StrepTagII and Flag tags; LUC, coding sequence for luciferase. SV40 pA, polyadenylation signal; RPBSA, constitutive promoter; GFP, Green Fluorescence Protein reporter gene; rtTA, reverse Tetracycline transactivator gene; PURO, puromycin selection gene; bGH pA, polyadenylation signal (Modified from Kowarz *et al.*, 2015). (B) Representative images of U251-MG-M2-KO cells transfected with the pSBtet-GP-MCPIP2 vector 24 hours after transfection in phase contrast and fluorescence (GFP).

mature termination codon (Fig. 1C). Thus, clone K5 was used for further experiments and was modified by the *Sleeping Beauty* system.

Generation of a U251-MG cell line with inducible overexpression of MCPIP2 protein

MCPIP2 is the least known member of the MCPIP protein family which participates in regulation of inflammation through degradation of inflammatory transcripts and modification of cell signaling pathways. Our data indicate that it possesses an RNase activity, however, its molecular targets are largely unknown (Wawro *et al.*, 2019). Our aim was to identify RNA species regulated by MCPIP2. In order to do that, we developed a stable U251-MG cell line with inducible overexpression of MCPIP2 using the SB transposon system. The genetic construct was prepared on the basis of the pSBtet-GP vector (Fig. 2A). This vector contains an expression cassette encoding MCPIP2 flanked by SB ITRs which allows its integration into the genome in the presence of the SB transposase, and results in generation of a stable cell line with inducible overexpression of MCPIP2 (pSBtet-GP-MCPIP2). Expression cassette in pSBtet-GP contains inducible and constitutive promoters that control transcription of different genes. A constitutive promoter (RPBSA) drives expression of the reporter protein (GFP), the selection marker (puromycin resistance gene) and rtTA protein. The last one in the presence of doxycycline (Dox) is capable of binding to the TRE (tetracycline response element) site that is present within the inducible promoter. This binding initializes expression of the downstream expression cassette (MCPIP2 or luciferase). Integration of the expression cassette into the genome is possible in the presence of a transposase, which is encoded by a separate vector called

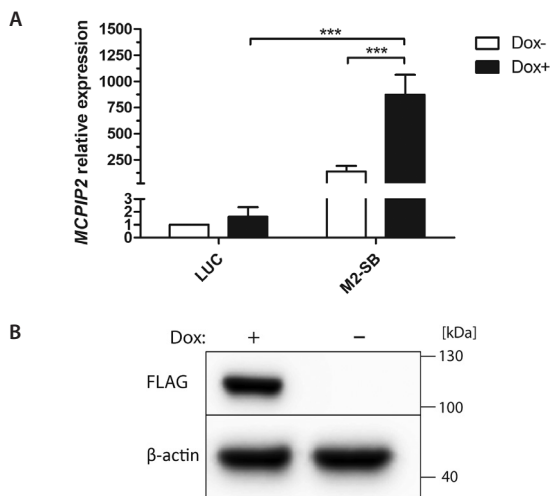


Figure 3. Analysis of MCPIP2 expression in the U251-MG-M2-SB cell line.

(A) *MCPIP2* mRNA level in U251-MG-M2-SB (M2-SB) and U251-MG-LUC (LUC) cells. Cells were treated with doxycycline (Dox+) or left untreated (Dox-) for 24 hours, and the level of *MCPIP2* mRNA was measured by real-time PCR. The plot shows mean results from three independent experiments ($n=3$) \pm S.D., *** $p < 0.001$. Two-way ANOVA with Bonferroni's post-hoc test was used. (B) *MCPIP2* expression in U251-MG-M2-SB (M2-SB) cells. Cells were treated with doxycycline (Dox+) for 24 hours or left untreated (Dox-) and *MCPIP2* protein level was examined by Western Blot analysis with anti-FLAG antibody. β -actin was used as a loading control (lower panel).

pCMV(CAT)T7-SB100 (pCMV-SB100X). Both vectors have to be co-delivered into the cells during transfection in the right proportions. A higher amount of vector encoding transposase results in more copies of transgene being integrated into the genome. Thanks to the presence of the selection marker and reporter GFP protein, efficiency of transfection can be assessed by simple observation of GFP fluorescence under a microscope, and non-transfected cells can be eliminated by the presence of puromycin (Fig. 2B). To avoid different genetic backgrounds, modified cell line (U251-MG-M2-KO) was used for genome editing by the SB transposon system. Within a week we were able to obtain a stable cell line with inducible overexpression of the *MCPIP2* protein (U251-MG-M2-SB cell line), which was confirmed by real-time PCR and Western Blot analysis (Fig. 3A, B). In parallel, we have also generated a stable cell line with inducible overexpression of luciferase, which was obtained by transfection of U251-MG-M2-KO cells with the pSBtet-GP vector (U251-MG-LUC).

Sleeping Beauty system itself influences changes in the level of transcripts that are known *MCPIP2* targets

Using U251-MG-M2-SB cells with inducible overexpression of *MCPIP2* and U251-MG-M2-KO cells with *MCPIP2* gene knockout, we decided to examine the level of transcripts that are known to be *MCPIP2* targets. We also examined the level of targets which are not regulated by this RNase (Wawro *et al.*, 2019). As a negative control, we have used the U251-MG-LUC cell line with inducible expression of luciferase. Recently, we have shown that *IL-6* mRNA possesses a stem-loop structure in its 3'UTR which is recognized by *MCPIP2* and is involved in the interaction between *MCPIP2* and this transcript, followed by mRNA degradation (Wawro *et al.*, 2019). Indeed, we observed a decrease in *IL-6* mRNA level in cells expressing *MCPIP2* after induction with

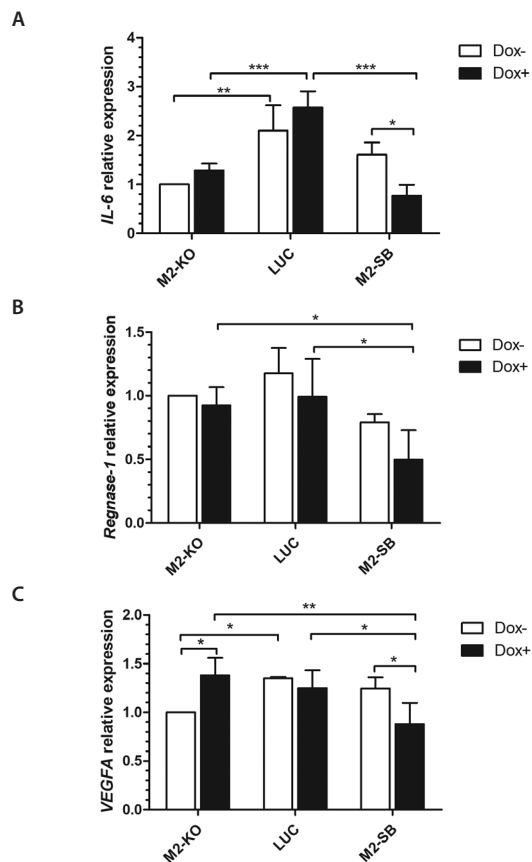


Figure 4. Influence of Sleeping Beauty system and *MCPIP2* over-expression on the level of *IL-6*, *Regnase-1* and *VEGFA* mRNA.

(A) *IL-6* (B) *Regnase-1* (C) *VEGFA* mRNA expression profiles in U251-MG-M2-KO cells (M2-KO), U251-MG-LUC (LUC) or U251-MG-M2-SB (M2-SB) with inducible overexpression of *MCPIP2* or luciferase. Cells were treated with doxycycline (Dox+) for 24 hours or left untreated (Dox-) and the levels of the mRNAs of interest were measured by real-time PCR analysis. The plots show mean results from three independent experiments ($n=3$) \pm S.D., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Two-way ANOVA with Bonferroni's post-hoc test was used.

Dox (Fig. 4A, M2-SB Dox+) in comparison to the control cells expressing luciferase (Fig. 4A, LUC Dox+). A similar pattern of regulation was observed for *Regnase-1* mRNA (Fig. 4B, LUC Dox+ and M2-SB Dox+). However, the level of transcripts that are not regulated by *MCPIP2*, such as *VEGFA* mRNA, is also influenced by the Dox-induced *MCPIP2* overexpression (Fig. 4C, LUC Dox+ and M2-SB Dox+). Thus, the system influences gene expression in *MCPIP2*-independent manner. Moreover, we have also observed an increase in the level of *IL-6* mRNA in the control U251-MG-LUC cells, in comparison to its level in the U251-MG-M2-KO cells (Fig. 4A). This suggests that the system itself influences changes in the level of *IL-6* mRNA. This phenomenon is not reflected by changes in the level of all investigated transcripts. The level of *Regnase-1* mRNAs is not influenced by SB modification of the U251-MG-M2-KO genome (Fig. 4B).

The observed stimulation of *IL-6* expression caused by transposon-mediated genome modification may be explained by specificity of the cell line used in our experiments. Astrocytoma is derived from astrocytes which belong to the facultative antigen-presenting cells. Following infection of the central nervous system with DNA viruses, astrocytes and microglia produce key anti-viral and inflammatory mediators, including *IL-6* (Van Wag-

oner & Benveniste, 1999). The DNA sensors such as DAI (DNA-dependent activator of interferon-regulatory factors) and cGAS (cyclic GMP-AMP synthase) have been identified in murine glial cells (Jeffries & Marriott, 2018). Thus, transfection of astrocytes with foreign DNA will result in a proinflammatory response. However, edition of the genome by the SB system is stable, the transcripts level was analyzed in stable-transfected cells after selection of positive/modified cells (puromycin resistance). Therefore explanation of the observed changes in IL-6 mRNA level (and lack of changes in Reg-1 and VEGFA mRNA level) in the SB-modified control cells is debatable. Taken together, our results indicate that the *Sleeping Beauty* approach, although very fast, efficient and versatile, may not be the best method for investigating changes in the transcriptome since it induces changes in the level of some transcripts.

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